SPECIFICATION

INS A1

Reshaped Human Antibody to Human Medulloblastoma Cells

TECHNICAL FIELD

The present invention relates to a human/mouse chimeric antibody comprising variable regions (V regions) of a mouse monoclonal antibody ONS-M21 to human medulloblastoma cells and constant regions (C regions) of human antibody; a reshaped human antibody wherein the complementarity determining regions (CDRs) of human light chain (L chain) V region and human heavy chain (H chain) V region, are substituted by the CDRs of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells; and the L chain or H chain that composes that antibody.

Moreover, the present invention provides a DNA coding for the above-mentioned antibody, and particularly its V region. Moreover, the present invention relates to a vector containing the above-mentioned DNA, and particularly to an expression vector, along with a host transformed with said vector. Moreover, the present invention provides a process for producing chimeric antibody to human medulloblastoma cells as well as a process for producing a reshaped human antibody to human medulloblastoma cells.

In addition, the present invention relates to a single chain Fv composed by linking an H chain V region of a reshaped human antibody to human medulloblastoma cells and an L chain V region of said antibody. In addition, the present invention relates to a process for producing the above-mentioned single chain Fv by using DNA coding for said single chain Fv, a recombinant vector comprising said DNA, and a host transformed with said recombinant vector.

BACKGROUND ART

Medulloblastoma is one type of primitive neuroectodermal tumors which account for 40% of all brain tumors with glioma, and is a malignant brain tumor that frequently appears in children under 10 years of age, and

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particularly between the ages of 5 to 10 y ars. This tumor is a typical undifferentiated tumor that mimics morphology nd arrangement patt rns of undifferentiated cells that compose th embryonal m dull ry epithelium and matrical layer. It is considered to b an immature tumor that has the potential to differentiate into both nerve cells and glia cells (Tamura, K. et al., Cancer Res., 49, 5380-5384 (1989)). Since these types of malignant tumors exhibit sensitivity to radiation and chemotherapeutic agents, it is treated by radiotherapy, chemotherapy as well as surgery.

However, although these treatment methods alleviate symptoms temporarily, there are many cases of relapse and death within several years, with an average survival period being 15 months. The cause of this is believed to be that a recurrent cancer has resistance to chemotherapy and radiation.

On the other hand, accurate evaluation of brain tumors relies heavily on histological techniques, and requires an extremely high level of specialized knowledge as well as auxiliary diagnostic technology.

Thus, there is a pressing need for the development of diagnostic tools and therapeutic drugs that enable early diagnosis and fundamental treatment.

Several attempts have been made at treatment through the use of monoclonal antibodies that recognize medulloblastoma in the past as well (refer to Kemshead, J.T. et al., Int. J. Cancer, 31, 187-195 (1983), Allan, P.M. et al., Int. J. Cancer, 31, 591-598 (1983), Jones, D. et al., Br. J. Hematol., 57, 621-631 (1984), Gross, N. et al., Cancer Res., 46, 2998-2994 (1986), Wikstrand, C.D. et al., Cancer Res., 46, 5933-5940 (1986), Gibson, F.M. et al., Int. J. Cancer, 39, 554-559 (1987), Feickert, H.J. et al., Cancer Res., 49, 4338-4343 (1989), Jennings, M.T. et al., J. Neurol. Sci., 89, 63-78 (1989) and Takahashi, H. et al., Neurosurg., 27, 97-102 (1990)).

However, since nearly all of these antibodies also recognize normal tissue and other tumors, they have the

disadvantage of being inappropriate for diagnosis and treatment of medulloblastoma.

Recently, brain tumor immunotherapy has been reported that uses a monoclonal antibody of human origin that reacts to the human glioma and exhibits ADCC activity (see, Japanese Unexamined Patent Publication No. 58-201994, Japanese Unexamined Patent Publication No. 59-137497 and Japanese Unexamined Patent Publication No. 4-356792).

Mouse monoclonal antibody exhibits a high degree of immunogenicity (also referred to as antigenicity) in humans. For this reason, their therapeutic value in humans is limited. Moreover, not only do mouse antibodies inhibit anticipated effects, but they cannot be administered frequently without provoking an immune reaction that brings about the risk of an allergic response that presents a problem for patients.

In order to solve these problems, a process for producing humanized antibody has been developed. Mouse antibody can be humanized by two methods. The simpler method involves the production of chimeric antibody wherein the variable region is derived from an original mouse monoclonal antibody, while the constant region is derived from a suitable human antibody. The resulting chimeric antibody contains a complete variable region of the original mouse antibody, and can be expected to bind antigen with the same specificity as the original mouse antibody.

Moreover, since the ratio of the protein sequence derived from sources other than humans is essentially reduced, it is predicted to have a low level of immunogenicity in comparison with the original mouse antibody. Although chimeric antibody effectively binds with antigen and has a low level of immunogenicity, there is still possibility of an immune reaction to the mouse variable region (LoBuglio, A.F. et al., Proc. Natl. Acad. Sci. USA, 86, 4220-4224, 1989).

Although more complex, the second method for humanizing mouse antibody considerably lowers the potential immunogenicity of the mouse antibody ven mor . In this m thod, complementarity determining regions (CDRs) from variable regions of a mouse antibody are transplanted into human antibody variable regions to produce a reshaped human antibody variable regions.

Next, these reshaped human antibody variable regions are linked to human antibody constant regions. Ultimately, the portion derived from the non-human protein sequence of a reshaped human antibody is only the CDRs and an extremely small portion of the framework (FR). CDRs comprise hyper-variable protein sequences. These sequences do not exhibit type-specific sequences. For these reasons, a reshaped human antibody containing mouse CDRs ought not to have immunogenicity stronger than naturally-occuring human antibody containing human CDRs.

The following references should be referred to with respect to reshaped human antibodies: Riechmann, L. et al., Nature, 332, 323-327, 1988; Verhoeyen M. et al., Science, 239, 1534-1536, 1988; Kettleborough, C.A. et al., Protein Engng., 4, 773-783, 1991; Maeda, H. et al., Human Antibodies and Hybridoma, 2, 124-134, 1991; Gorman S.D. et al., Proc. Natl. Acad. Sci. USA, 88, 4181-4185, 1991; Tempest P.R. et al., Bio/Technology, 9, 266-271, 1991; Co, M.S. et al., Proc. Natl. Acad. Sci. USA, 88, 2869-2873, 1991; Carter, P. et al., Proc. Natl. Acad. Sci. USA, 89, 4285-4289, 1992; Co, M.S. et al., J. Immunol., 148, 1149-1154, 1992; and, Sato, K. et al., Cancer Res., 53, 851-856, 1993.

As was previously stated, although it is predicted that a reshaped human antibody is useful for the purpose of therapeutical treatment, a reshaped human antibody to human medulloblastoma cells is not known. Moreover, there are no methods for producing a reshaped human antibody that can be universally applied to any specific antibody. Thus, various contrivances are necessary to produce a reshaped

human antibody to a specific antigen that has sufficient activity (for example, Sato, K. et al., Cancer Res., 53, 1-6 (1993)).

The inventors of the present invention isolated and established a medulloblastoma cell line (ONS-76) from the cerebellum of medulloblastoma patients (Tamura, K. et al., Cancer Res., 49, 5380-5384 (1989)). By then immunizing mice with said medulloblastoma cell line ONS-76, a mouse monoclonal antibody (ONS-M21) was found that specifically recognizes human medulloblastoma but does not cross-react with normal brain tissue or peripheral blood cells (Moriuchi, S. et al., Br. J. Cancer, 68, 831-837 (1993)). Since antigen recognized by this antibody is strongly expressed in brain tumors such as medulloblastoma and some gliomas, it is anticipated to be used as a diagnostic tool as well as directly destroy cancer cells by inducing ADCC and CDC or conjugating with toxins and radioisotopes.

DISCLOSURE OF THE INVENTION

Thus, the present invention relates to a reshaped human antibody of a mouse monoclonal antibody ONS-M21 to human medulloblastoma cells. In addition, the present invention provides a human/mouse chimeric antibody useful during the course of producing said reshaped human antibody. Moreover, the present invention relates to a genetic engineering process for producing a reshaped human antibody and a chimeric antibody of mouse monoclonal antibody ONS-M21.

More specifically, the present invention relates to

(1) L chain V region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells; and,

(2) H chain V region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells.

The present invention also relates to a chimeric antibody to human medulloblastoma cells comprising:

(1) L chain containing a human antibody L chain C region, and the above-mentioned L chain V region of mouse

monoclonal antibody ONS-M21 to human medulloblastoma cells; and,

(2) H chain containing human antibody H chain C region, and the H chain V region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells.

The present invention moreover relates to a reshaped human antibody of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells comprising:

reshaped human L chain V region comprising

(1) framework regions (FRs) of human antibody L chain V region, and

(2) CDRs of L chain V region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells; and,

reshaped human H chain V region comprising

(1) FRs of human antibody H chain V region, and

(2) CDRs of H chain V region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells.

In addition, the present invention relates to L chain or H chain polypeptides that compose the various above-mentioned antibodies as well as DNA coding for them.

Moreover, the present invention relates to expression vectors containing the above-mentioned DNA as well as a host transformed by them.

In addition, the present invention relates to a process for producing chimeric antibody to human medulloblastoma cells as well as a process for producing a reshaped human antibody to human medulloblastoma cells.

In addition, the present invention relates to a single chain Fv region composed by linking an H chain V region of a reshaped human antibody to human medulloblastoma cells and an L chain V region of said monoclonal antibody.

Moreover, the present invention relates to DNA coding for the above-mentioned single chain Fv region, recombinant vectors that contain said DNA and a host transformed by said recombinant vectors.

Moreover, the present invention relates to a process for producing a single chain Fv region characterized by culturing the above-mentioned host and recovering a single chain Fv region from said culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 indicates expression plasmids HEF-VL-gκ and HEF-VH-gγ1 comprising human EF1-α promoter/enhancer useful for expression of an L chain and H chain, respectively.

Fig. 2 is a graph indicating the binding ability of chimeric antibody ONS-M21 (ChM21) to human medulloblastoma cell line ONS-76.

Fig. 3 is a diagram of the preparation of the first version (Version "a") of the L chain V region of reshaped human antibody ONS-M21.

Fig. 4 is a diagram of the preparation of the H chain V region of reshaped human antibody ONS-M21.

Fig. 5 is a graph indicating the binding ability of antibody comprising reshaped human H chain and chimeric L chain to human medulloblastoma cell line ONS-76.

Fig. 6 is a graph comparing the binding ability of 8 types of reshaped human ONS-M21 antibodies comprising reshaped human H chain and one of versions "a" through "h" of reshaped human L chain, to human medulloblastoma cell line ONS-76, with that of chimeric antibody (ChM21).

Fig. 7 is a graph comparing the binding ability of 6 types of reshaped human ONS-M21 antibodies comprising the reshaped human H chain and one of versions "i", "j", "l", "m", "o" and "p" of reshaped human L chain of the present invention, to human medulloblastoma cell line ONS-76, with that of chimeric antibody (ChM21) and antibodies having reshaped L chain versions "k" and "n".

Fig. 8 is a schematic diagram of a preparation process of DNA coding for the single chain Fv region of the present invention.

Fig. 9 indicates the structure of an example of expression plasmids used for expressing DNA coding for the single chain Fv region of the present invention.

Fig. 10 is a graph that indicates an antigen binding activity of the single chain Fv region of the present invention (scFv-hM21) in comparison with an antigen binding activity of reshaped human ONS-M21 antibody and the Fab fragment of said antibody using for the parameter the inhibition of antigen binding by mouse monoclonal antibody ONS-M21.

DETAILED EXPLANATION

Cloning of DNA Coding for Mouse Antibody V Region

In order to clone a DNA coding for a V region of a mouse monoclonal antibody to human medulloblastoma cells, after preparing mRNA from mouse monoclonal antibody-producing cells, said mRNA is converted to double-stranded DNA using known method followed by amplifying the target DNA using polymerase chain reaction (PCR). It is necessary to prepare hybridoma producing a monoclonal antibody to human medulloblastoma cells for the supply source of mRNA. One example of this type of hybridoma is ONS-M21. The process for producing hybridoma ONS-M21 is described later in Reference Example 1.

(1) Obtaining of Whole RNA

In order to obtain whole RNA, in the present invention, after destroying hybridoma cells and treating with guanidine thiocyanate, cesium chloride density gradient centrifugation was performed (Chirgwin, J.M. et al., Biochemistry, 18, 5294-5299, 1979). However, methods already used during cloning of other protein genes can also be used, examples of which include treatment with surfactant in the presence of a ribonuclease inhibitor such as vanadium compounds followed by treatment with phenol (Berger, S.L. et al., Biochemistry, 18, 5143-5149, 1979).

(2) Preparation of Double-Stranded cDNA

In order to obtain single-stranded DNA from whole RNA obtained in the manner described above, single-stranded DNA complementary to whole RNA (cDNA) can be synthesized by using the whole RNA as a template and treating with reverse transcriptase using oligo(dT)

complementary to its polyA chain on the 3' terminal as primer (Larrik, J.W. t al., Bio/Technology, 7, 934-938, 1989). In addition, a random primer may also be used at that time.

(3) Amplification of Mouse Antibody V R gion by Polymerase Chain Reaction (PCR)

Next, specific amplification of mouse antibody V region is performed from the above-mentioned cDNA using polymerase chain reaction (PCR). The primer described in Jones, S.T. et al., Bio/Technology, 9, 88-89, 1991 can be used for amplification of the mouse antibody V region. In the determining of the primer used for cloning of mouse monoclonal antibody ONS-M21 produced by hybridoma ONS-M21, it is necessary to type the H chain and L chain and determine the form of both chains.

As a result of performing typing of ONS-M21 antibody using a mouse monoclonal antibody isotyping kit (Amersham International Plc.), it was clear that ONS-M21 antibody has a Cκ type L chain and γ-1C type H chain. Typing of ONS-M21 is described later in Reference Example 2.

Next, in order to amplify the kappa (κ) type L chain V region of mouse monoclonal antibody using polymerase chain reaction (PCR), 11 types of oligonucleotide primers indicated in SEQ ID Nos: 1 to 11 (Mouse Kappa Variable; MKV) and the oligonucleotide primer shown in SEQ ID NO: 12 (Mouse Kappa Constant; MKC) are used for the 5'-terminal primer and 3'-terminal primer, respectively.

The above-mentioned MKV primer hybridizes with the DNA sequence coding for the mouse kappa type L chain leader sequence, while the above-mentioned MKC primer hybridizes with the DNA sequence coding for the mouse kappa type L chain C region.

In order to amplify the H chain V region of mouse monoclonal antibody, the 12 types of oligonucleotide primers indicated in SEQ ID NOs: 13 to 24 (Mouse Heavy Variable; MHV) and the oligonucleotide indicated in SEQ ID

NO: 25 (Mouse Heavy Constant, MHC) are used for the 5'-terminal primer and 3'-terminal primer, respectively.

Furthermore, in the present embodiment, a 5'-terminal primer contains the sequence GTCGAC that provides a site to be cleaved by restriction enzyme SalI near the 5'-terminal, while a 3'-terminal primer contains the nucleotide sequence CCCGGG that provides a site to be cleaved by restriction enzyme XmaI near the 5'-terminal. Other restriction enzyme cleavage sites may be used for these restriction enzyme cleavage sites provided they can be used for subcloning the target DNA fragment coding for the variable region in a cloning vector.

Next, in order to obtain a DNA fragment coding for a target variable region of a mouse monoclonal antibody, after cleaving the amplification product with restriction enzymes SalI and XmaI, isolation and purification is performed using low melting temperature agarose or a column (PCR product purification kit (Qiagen), DNA purification kit (Geneclean II) and so forth). On the other hand, a plasmid is obtained that contains a DNA fragment coding for a target variable region of mouse monoclonal antibody by cleaving a suitable cloning vector such as plasmid pUC19 with the same restriction enzymes SalI and XmaI, and ligating the above-mentioned DNA fragment to this pUC19.

The cloned DNA can be sequenced using any commonly employed method such as an automated DNA sequencer (Applied Biosystems Inc.).

Cloning of the target DNA and determination of its sequence are described in detail in Examples 1 and 2.

Complementarity Determining Region (CDR)

A pair of V regions of L chain and H chain forms an antigen binding site. The variable regions of the L chain and H chain comprises four relatively well-preserved framework regions linked with three hyper-variable or complementarity determining regions (CDR) (Kabat, E.A. et al., "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983).

The majority of the portions of th above-mentioned four framework regions (FRs) employ a β-sheet structure. As a result, thr CDRs form a loop. The CDRs may also form a portion of the β-sheet structur in certain cases. The three CDRs are maintained at positions that are three-dimensionally extremely close to each other by the FRs, and contribute to the formation of an antigen binding site together with three CDRs of the region with which it constitutes a pair.

These CDRs can be found based on the empirical rules of Kabat, E.A. et al., "Sequences of Proteins of Immunological Interest" by comparing the amino acid sequence of the V region of the resulting antibody with known amino acid sequences of V regions of known antibodies. A detailed explanation of this is given in Example 3.

Production of Chimeric Antibody

Prior to designing the reshaped human V region of antibody to human medulloblastoma cells, it is necessary to confirm that the CDRs used actually form an antigen binding region. Chimeric antibody was produced for this purpose. Moreover, the amino acid sequence of mouse anti-human medulloblastoma cell antibody, predicted from the nucleotide sequence of cloned DNA of monoclonal antibody ONS-M21 described in Example 1, was compared with the V regions of known mouse and human antibodies.

Once a DNA fragment is cloned coding for the mouse L chain and H chain V regions of monoclonal antibody ONS-M21, chimeric anti-human medulloblastoma cell antibody can be obtained by linking these mouse V regions with DNA coding for human antibody constant region and then expressing them.

The basic method for producing chimeric antibody comprises linking a mouse leader sequence and V region sequence in cloned cDNA with a sequence coding for human antibody C region already present in a mammalian cell expression vector.

The above-mentioned human antibody C region can be any human L chain C region or H chain C region, examples of which include human L chain Cκ or H chain γ-1C and γ-4C.

In order to produce chimeric antibody, two types of expression vectors are prepared. These vectors are an expression vector comprising DNA coding for a mouse L chain V region and a human L chain C region under the control by an expression control region such as an enhancer/promoter type, and an expression vector comprising DNA coding for a mouse H chain V region and a human H chain C region under the an expression control region such as an enhancer/promoter type. Next, host cells such as mammalian cells are co-transformed with these expression vectors after, which the transformed host is cultured in vitro or in vivo to produce chimeric antibody (e.g. WO91-16928).

Alternatively, DNA coding for a mouse L chain V region and a human L chain C region, and DNA coding for a mouse L chain V region and a human H chain C region may be introduced into a single expression vector, host cells are transformed using said vector, and this transformed host is then cultured in vivo or in vitro to produce the desired chimeric antibody.

A description of production of chimeric antibody is provided in Example 4.

A cDNA coding for a mouse ONS-M21 κ type L chain leader region and a V region is subcloned using PCR and linked to an expression vector containing DNA coding for human genome L chain Cκ chain region. cDNA coding for the γ1 type H chain leader and V regions of mouse ONS-M21 antibody is subcloned using PCR, and linked to an expression vector containing genome DNA coding for human γ-1C region.

Using specially designed PCR primers, cDNA coding for the V region of mouse ONS-M21 is provided with a suitable nucleotide sequence at its 5'-terminal and 3'-terminal to facilitate their insertion into the expression vector as well as to ensure that they function suitably in said

expression vector (for example, transcription efficiency is improved in the present invention by the introduction of Kozak's sequence). Next, the V region of mouse ONS-M21, obtained by amplification by PCR, was inserted into an HEF expression vector (Fig. 1) already containing the desired human C region using these primers. These vectors are suitable for transient or stable expression of genetically engineered antibodies in various mammalian cell systems.

The chimeric ONS-M21 antibody demonstrated an activity to bind to human medulloblastoma cells. Thus, this indicated that a correct mouse V region had been cloned, and that the sequence had been determined.

Design of Reshaped Human ONS-M21 Antibody V Region

In order to produce a reshaped human antibody in which the CDRs of mouse monoclonal antibody are grafted onto a human antibody, it is preferable that a high degree of homology exists between the FRs of the mouse monoclonal antibody and the FRs of the human antibody. Thus, the V regions of the L chain and H chain of mouse ONS-M21 antibody were compared with the V regions of all known antibodies for which structure has been determined using the Protein Data Bank.

The L chain V region of mouse ONS-M21 most closely resembles the consensus sequence of subgroup IV of human L chain V region (HSGIV), demonstrating homology of 61.9%.

In a comparison of the L chain V region of mouse ONS-M21 antibody with known human antibody L chain V regions, homology of 60.4% was demonstrated with human L chain V region REI, a member of subgroup I of the human L chain V region. Thus, the FRs of REI were used as a starting material for preparation of reshaped human ONS-M21 antibody L chain V region.

Sixteen versions of reshaped human ONS-M21 antibody L chain V region were designed (versions "a"-"p"). In the first version (version "a"), human FRs were identical to FR of version "a" of the L chain V region of reshaped human PM-1 described in WO92-19759, which is based on REI present

in reshaped human CAMPATH-1H antibody (Riechmann, L. et al., Nature, 332, 323 - 327 (1988)), while mouse CDRs were identical to the CDRs in the L chain V region of mouse ONS-M21 antibody.

Tables 1 and 2 show the amino acid sequences of the L chain V region of mouse ONS-M21 antibody, the FRs of REI, and the L chain V region of the 16 versions of reshaped ONS-M21 antibody.

Table 1

Design of Reshaped Human ONS-M21 L Chain V Regions

	FR1		CDR1	FR2	CDR2
	1	2	3	4	5
	12345678901234567890123		45678901234	567890123456789	0123456
ONS-M21VL	DIVMTQSQKFMSTSVGDRVSVTC		KASQNVGTNVA	WYQQKPGQSPKPLIY	SASYRYS
RE	DIQMTQSPSSLSASVGDRVTITC			WYQQKPGKAPKLLIY	
RVL	DIQMTQSPSSLSASVGDRVTITC		KASQNVGTNVA	WYQQKPGKAPKLLIY	SASYRYS
RVLb	———————————		———————	———————————	———————
RVLc	———————————		———————	———————————	———————
RVLd	———————————		———————	———————————	———————
RVLe	——————————SV—		———————	———————————	———————
RVLf	——————————SV—		———————	———————————	———————
RVLg	——————————SV—		———————	———————————	———————
RVLh	———————————		———————	———————————	———————
RVLi	——————————SV—		———————	————QS—P——	———————
RVLj	——————————SV—		———————	————QS—P——	———————
RVLk	——————————SV—		———————	———————————	———————
RVLl	——————————SV—		———————	————QS—P——	———————
RVLm	———————————		———————	————QS—P——	———————
RVLn	———QKF——————		———————	————QS———	———————
RVLo	———————————		———————	————QS—P——	———————
RVLp	———————————		———————	——————P——	———————

Table 2

Design of Reshaped Human ONS-M21 L Chain V Regions (cont.)

		FR3			CDR3	FR4
		6	7	8	9	10
		7890123456789012345678			901234567	8901234567
A20	ONS-M21VL	GVPDRFTGSGSGTDFTLTITNVQSEDLADYFC			QQYNSYPRA	FGGGTKLEIK
A21	REI	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC				FGQGTKVEIK
A22	RVLa	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC			QQYNSYPRA	FGQGTKVEIK
A23	RVLb	——————Y——————————			———————	———————
A24	RVLc	——————————————————F-			———————	———————
A25	RVLd	——————Y———————F-			———————	———————
A26	RVLe	———————————————————			———————	———————
A27	RVLf	——————————————————F-			———————	———————
A28	RVLg	————————L———————F-			———————	———————
A29	RVLh	————————L———————			———————	———————
A30	RVLi	————————L———————F-			———————	———————
A31	RVLj	————————L———————D-F-			———————	———————
A32	RVLk	————————L———————D-F-			———————	———————
A33	RVLl	———————————————————			———————	———————
A34	RVLm	———————————————————			———————	———————
A35	RVLn	———————————————————			———————	———————
A36	RVLo	———————————————————			———————	———————
A37	RVLp	———————————————————			———————	———————

Note: Those underlined amino acids in the FRs of REI indicate locations of amino acids that differ from those of the amino acid sequence of human REI (Palm, W. et al., Hoppe-Seyler's, Z. Physiol. Chem., 356, 167-191, 1975).

The H chain V region of mouse ONS-M21 most closely resembles the consensus sequence of human H chain V region subgroup I (HSGI), exhibiting homology of 57.9%. In a comparison of the H chain V region of mouse ONS-M21 antibody with known human antibody H chain V regions, it resembled extremely closely the H chain V region of human antibody Eu, a member of human H chain V region subgroup I, from FR1 to FR3 (Cunningham, B.J. et al., Biochemistry, 9, 3161, 1970). Moreover, the size of the CDRs were also extremely similar between mouse ONS-M21 antibody and human antibody Eu.

Consequently, the FRs of human antibody Eu were used as a starting material for preparation of the H chain V region of a reshaped human ONS-M21 antibody.

However, since the amino acid sequence of the FR4 of the human antibody Eu has a sequence that differs from the human antibody subgroup I consensus sequence, it was decided to use the amino acid sequence of the FR4 of human antibody ND (Kenten, J.H. et al., Proc. Natl. Acad. Sci. USA, 79, 6661-6665 (1982)), whose V region belongs to subgroup I, for the FR4 in this case.

H chain V regions of reshaped human ONS-M21 antibody were designed. Amino acids at positions 27, 28, 29 and 30 of human FR1 and position 94 of FR3 were made to be identical to the amino acids of mouse ONS-M21.

Table 3

Design of Reshaped Human ONS-M21 H Chain V Region

		FR1			CDR1	FR2
		1	2	3		4
		12345678901234567890123456789			12345	67890123456789
Dur A38 (SEQ ID NO:113)	ONS-M21VH	EVQLQQSGAELVKPGASVKLSCTASGFNIK			DTYIH	WAKQRPEQGLEWIG
	EU	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS				WVRQAPGQGLEWMG
pa. A89	RVHa	QVQLVQSGAEVKKPGSSVKVSCKASGFNIK			DTYIH	WVRQAPGQGLEWMG

		CDR2		FR3		
		5	6	7	8	9
		01223456789012345		678901234567890122223456789012 34		
		A			ABC	
	ONS-M21VH	RIDPADGNTKYDPKFQG		KATITADTSSNTAYLQLSSLTSEDTAVYYCAS		
	EU			RVTITADESTNTAYMELSSLRSEDTAFYFCAG		
	RVHa	RIDPADGNTKYDPKFQG		RVTITADESTNTAYMELSSLRSEDTAFYFCAS		

		CDR3	FR4
		1	1
		0	1
		56789012	34567890123
	ONS-M21VH	AYYVNQDY	WGQGTSVTVSS
	EU		
A40	ND		WGQGTTVTVSS
	RVHa	AYYVNQDY	WGQGTTVTVSS

Production of Reshaped Human ONS-M21 Antibody

Production of reshaped human ONS-M21 antibody V region is described in detail in Example 5.

A reshaped human ONS-M21 antibody of the present invention comprises:

(A) L chains lack comprising:

(1) a human L chain C region, and

(2) an L chain V region comprising human L chain FRs and L chain V region CDRs of mouse monoclonal antibody ONS-M21 to human medulloblastoma; and,

(B) H chains lack comprising:

(1) a human H chain C region, and

(2) an H chain V region comprising human H chain FRs and H chain V region CDRs of mouse monoclonal antibody ONS-M21 to human medulloblastoma.

In order to produce a reshaped human antibody that possesses sufficient activity with respect to a specific antigen, it is preferable to substitute a portion of the amino acid sequence of the above-mentioned human FRs.

In a preferable embodiment, the amino acid of position 46 of FR2 of the above-mentioned L chain V region should be proline, or the amino acids of positions 42, 43 and 46 should preferably be amino acids derived from mouse FR such as glutamine, serine and proline, or more preferably, should have the amino acid sequence of RVLi, RVLj, RVLl, RVLm, RVLo or RVLp in Tables 1 and 2.

The above-mentioned human L chain C region can be any human L chain C region, an example of which is the human κC region. The above-mentioned H chain C region can also be any human H chain C region, examples of which include the human γ-1C and human γ-4C regions. Alternatively, a toxin or radioisotope may be bound instead of the above-mentioned human L chain C region and/or above-mentioned human H chain C region.

In order to produce a reshaped human antibody, two types of expression vectors are prepared. These are an expression vector containing DNA coding for a previously defined reshaped human L chain under an expression control region such as an enhancer/promoter type, and another expression vector that contains DNA coding for a previously defined reshaped human H chain under an expression control region such as an enhancer/promoter type. Next, host cells such as mammalian cells are simultaneously transformed using these expression vectors after which the transformed host is cultured in vitro or in vivo to produce a reshaped human antibody (e.g. WO91-16927).

Alternatively, DNA coding for a reshaped L chain and DNA coding for a reshaped human H chain may be introduced into a single expression vector, host cells are transformed

with this vector, and this transformed host is then cultured in vivo or in vitro to produce a desired reshaped human antibody.

Moreover, Fab or Fv, or Fv-linked single-chain Fv, can be produced in a suitable host and used for the purpose described above (refer, for example, to Bird, et al., TIBTECH, 9, 132-137 (1991)).

Chimeric antibody or humanized antibody produced by culturing a transformed host transformed with a gene coding for the desired chimeric antibody or humanized antibody in the manner described above can then be isolated from inside or outside cells and purified to homogeneity.

Furthermore, separation and purification of the chimeric antibody or humanized antibody, which is the target protein of the present invention, can be performed using a protein A agarose column. In addition, other isolation and purification methods commonly used with proteins may also be used, and there are no limitations whatsoever on those methods. For example, chimeric antibody or humanized antibody can be isolated and purified by suitably selecting and combining various types of chromatography, ultrafiltration, salting, dialysis and so forth.

Any expression system can be used for producing a chimeric antibody or a reshaped human antibody to human medulloblastoma cells of the present invention, examples of which include eucaryotic cells such as animal cells including established mammalian cell systems, mold cells and yeast cells as well as procaryotic cells such as bacterial cells including Escherichia coli cells. A chimeric antibody or a reshaped antibody of the present invention is preferably expressed in mammalian cells such as COS cells or CHO cells.

In these cases, useful, conventional used promoters can be used for expression in mammalian cells. For example, the use of human cytomegalovirus immediate early (HCMV) promoter is preferable. Examples of expression

vectors containing HCMV promoter include HCMV-V_H-HCγ1 and HCMV-V_L-HCK derived from pSV2neo (ref r to International Unexamin d Application WO92-19759).

In addition, promoters derived from mammalian cells such as promoters of virus s including retrovirus, polio virus, adenovirus and simian virus 40 (SV40) as well as human polypeptide chain elongation factor 1α (HEF-1α) should be used for promoters of genetic expression in mammalian cells that can be used for the present invention. For example, in the case of using SV40 promoter, expression can be easily carried out by following the method Mulligan, R.C. et al. (Nature, 277, 108-114 (1979)), or in the case of using HEF-1α promoter, the method of Mizushima, S. et al. (Nucleic Acids Research, 18, 5322 (1990)).

Those derived from SV40, polio virus, adenovirus, bovine papilloma virus (BPV) and so forth can be used for an origin of replication. Moreover, in order to amplify the number of gene copies in the host cell system, the expression vector can contain phosphotransferase APH(3')II or I (neo) gene, thymidine kinase (TK) gene, Escherichia coli xanthine-guanine phosphoribosyl transferase (Ecogpt) gene and dihydrofolic acid reductase (DHFR) and so forth as selection markers.

In addition, the present invention also provides a single-chain Fv composed by linking an H chain V region and an L chain V region of a reshaped human antibody to human medulloblastoma cells. The H chain V region and L chain V region in this scFv polypeptide are preferably linked by a linker, and more preferably, a peptide linker.

The H chain V region in the single-chain Fv may be any of the H chain V regions of reshaped human antibody previously described.

An H chain V region comprises 4 FRs, and 3 CDRs having the amino acid sequences defined below:

Jno. A4135

Jno. A42

A41 CDR1: Asp Thr Tyr Ile His

A42 CDR2: Arg Ile Asp Pro Ala Asp Gly Asn Thr Lys Tyr Asp Pro Lys Phe Gln Gly

CDR3: Ala Tyr Tyr Val Asn Gln Asp Tyr
or a portion ther of.

In addition, an L chain V region comprises 4 FRs, and 3 CDRs h ving the amino acid s qu nc s defined below:

CDR1: Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala
CDR2: Ser Ala Ser Tyr Arg Tyr Ser
CDR3: Gln Gln Tyr Asn Ser Tyr Pro Arg Ala,
or a portion thereof.

A specific example of a single-chain Fv is that comprising an H chain V region consisting of the amino acid sequence from amino acid 1 to 116 in the amino acid sequence described in SEQ ID NO: 99, and an L chain V region consisting of the amino acid sequence from amino acid 1 to 106 in the amino acid sequence described in any of SEQ ID NOs: ~~40, 43, 46, 47, 59, 51, 54, 55, 58, 61, 62, 63, 66, 69, 70, ... 72~~.

In addition, a preferable example of a single-chain Fv is that comprising an H chain V region consisting of the amino acid sequence from amino acid 1 to 116 in the amino acid sequence described in SEQ ID NO: 99, and an L chain V region consisting of the amino acid sequence from amino acid 1 to 106 in the amino acid sequence described in SEQ ID NO: 91.

These V regions are preferably linked by polypeptide linkers. Although examples of polypeptide linkers include any single-chain Fv comprising 12 to 19 amino acids, a specific example of a peptide fragment that can be used is the peptide fragment composed of Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO: 90).

An example of an amino acid sequence of a single-chain Fv is shown in SEQ ID NO: 109. A single-chain Fv that possesses this amino acid sequence is referred to as scFv-hM21 in the present invention, and is explained in detail in Example 6.

A DNA coding for the single-chain Fv of the present invention is obtained by using as a template a DNA coding for an H chain or H chain V region of a reshaped human ONS-

M21 antibody and a DNA coding for an L chain or L chain V region of reshaped human ONS-M21 antibody, both of which were previously explained in detail, and then amplifying a DNA portion coding for a desired amino acid sequence in those sequences by PCR using a pair of primers that specify both ends. Example 6 provides a detailed description of a method for preparing a DNA coding for single-chain Fv comprising an H chain V region and an L chain V region version "p". Since the amino acid sequences of L chain V region versions "a" to "o" along with methods for preparing DNA coding for them are described in detail, by applying a method in which version "p" is used for those versions, DNA can be produced that code for various single-chain Fvs of the present invention.

In addition, once DNA coding for single-chain Fv has been produced, expression vectors comprising that DNA as well as hosts transformed with said expression vectors can be obtained in accordance with routine methods. In addition, single-chain Fvs can be obtained by using those hosts in accordance with conventional methods. Specific examples of these are described in detail in Example 6.

As a result of comparing the antigen binding ability of scFv-hM21 with that of humanized ONS-M21 antibody and Fab fragment using for the indicator the degree of inhibition of binding of mouse ONS-M21 antibody to ONS-76 cells, scFv-hM21 was found to exhibit binding inhibition equal to that of Fab fragment. On the basis of the above, a single-chain Fv was able to be successfully constructed that possesses the same degree of affinity as the original antibody.

In general, since single-chain Fvs are considered to exhibit superior mobility into tissue and tumors in comparison with whole IgG, this successfully constructed scFv-hM21 is expected to be used in the future in imaging by RI labeling as well as a therapeutic drug by coupling with toxins or RI.

EXAMPLES

Next, although the following provides a specific explanation of the present invention through its Examples, the scope of the present invention is not limited by these Examples.

Example 1. Cloning of DNA Coding for the V Region of Mouse Monoclonal Antibody to Human Medulloblastoma Cells

DNA coding for a variable region of mouse monoclonal antibody ONS-M21 to human medulloblastoma cells was cloned in the following manner.

1. Preparation of Messenger RNA (mRNA)

mRNA from hybridoma ONS-M21 was prepared using the Fast Track mRNA Isolation Kit Version 3.2 (Invitrogen).

2. Synthesis of Double-Stranded cDNA

Double-stranded cDNA was synthesized using The Copy Kit (Invitrogen) from approximately 4 µg of mRNA.

3. Amplification of Gene Coding for Antibody Variable Region by PCR

PCR was performed using Thermal Cycler (Perkin Elmer Cetus).

(1) Amplification of Gene Coding for Mouse L Chain V Region

MKV (Mouse Kappa Variable) primers (Jones, S.T. et al., Bio/Technology, 9, 88-89 (1991)) shown in SEQ ID NOs: 1 to 11, which hybridize with mouse kappa type L chain leader sequence, were used for the primers used in PCR.

100 µl of PCR solution contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM $MgCl_2$, 5 units of DNA polymerase Ampli Taq (Perkin Elmer Cetus), 0.1 µM of MKV primer shown in SEQ ID NOs: 1 to 11, 0.4 µM of MKC primer shown in SEQ ID NO: 12 and 0.1 µg of double-stranded cDNA, and each of MCK primers 1 to 12 was separately amplified. After covering with 50 µl of mineral oil, the reaction mixture was heated at an initial temperature of 94°C for 3 minutes and then in the order of 94°C for 1 minute, 50°C for 1 minute and 72°C

for 1 minute. After repeating this temperature cycle 30 times, the reaction mixture was further incubated for 10 minutes at 72°C.

(2) Amplification of cDNA Coding for Mouse H Chain V Region

MHV (Mouse Heavy Variable) primers 1 to 12 shown in SEQ ID NOs: 13 to 24 and MHC-GI (Mouse Heavy Constant) primer (Jones, S.T. et al., Bio/Technology, 9, 88-89 (1991)) shown in SEQ ID NO: 25 were used for the primers for PCR.

Amplification of cDNA was performed according to the same method as that described for amplification of L chain V region gene in the above-mentioned section 3(1) with the exception of performing amplification using a mixture of 0.25 μM of each MHV primer and 2.5 μM of MHC-GI primer.

4. Purification and Cleavage of PCR Products

DNA fragments amplified by PCR as described above were purified with low melting temperature agarose (Sigma), and digested for 3 hours at 37°C using 5 units of restriction enzyme XmaI (New England Biolabs) in 10 mM Tris-HCl (pH 7.9) containing 10 mM $MgCl_2$ and 1 mM dithreitol.

Next, after digesting for 2 hours at 37°C with 40 units of restriction enzyme SalI (Takara Shuzo), the resulting DNA fragments were separated by agarose gel electrophoresis using 1.5% low melting temperature agarose (Sigma).

A piece of agarose containing a DNA fragment of approximately 450 bp in length was cut out, melted for 5 minutes at 65°C followed by the addition of an equal volume of 20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA and 200 mM NaCl. This mixture was extracted with phenol and chloroform, the DNA fragments were recovered by ethanol precipitation and then dissolved in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA.

Thus, a DNA fragment comprising a gene coding for a mouse kappa type L chain variable region, and a DNA fragment comprising a gene coding for a mouse H chain variabl region were obtained. The above-mentioned DNA fragments have a SalI-cohesive end on their 5'-terminal, and an XmaI-cohesive end on their 3'-terminal.

5. Linkage and Transformation

Approximately 0.3 µg of SalI-XmaI DNA fragments comprising a gene coding for a mouse kappa type L chain V region prepared in the above manner were linked with approximately 0.1 µg of pUC19 vector prepared by digesting with SalI and XmaI, by reacting for 4 hours at 16°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 50 mg/ml of polyethylene glycol (8000) and 1 unit of T4 DNA ligase (Gibco BRL).

Next, 10 µl of the above-mentioned linking mixture was added to 50 µl of E. coli DH5α competent cells after which the cells were allowed to stand undisturbed for 30 minutes on ice, 1 minute at 42°C and again for 1 minute on ice. Next, 400 µl of 2 x YT medium (Molecular Cloning: A Laboratory Manual, Sambrooks et al., Cold Spring Harbor Laboratory Press, 1989) was added, and after incubating for 1 hour at 37°C, 2 x YT agar medium (Molecular Cloning: A Laboratory Manual, Sambrooks et al., Cold Spring Harbor Laboratory Press, 1989) was inoculated with the E. coli and incubated overnight at 37°C to obtain E. coli transformants.

These transformants were cultured overnight at 37°C in 10 ml of 2 x YT medium containing 50 µg/ml of ampicillin, and plasmid DNA was prepared from the culture according to the alkaline method (Molecular Cloning: A Laboratory Manual, Sambrooks, et al., Cold Spring Harbor Laboratory Press, 1989).

A plasmid containing a gene coding for a mouse kappa type L chain V region derived from hybridoma ONS-M21 obtained in this manner was named pUC-M21-V_L.

A plasmid containing a gene coding for a mouse H chain V region derived from hybridoma ONS-M21 was made from SalI-XmaI DNA fragments in accordanc with the same method as that described above, and that plasmid was named pUC-M21-V_H.

Example 2. Determination of DNA Nucleotide Sequence

A nucleotide sequence of a cDNA coding region in the above-mentioned plasmid was determined in accordance with the protocol specified by the manufacturer using an automated DNA sequencer (Applied Biosystem Inc.) and the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystem Inc.).

A nucleotide sequence of a gene coding for an L chain V region of mouse ONS-M21 antibody contained in plasmid pUC-M21-V_L is shown in SEQ ID NO: 26. In addition, a nucleotide sequence of a gene coding for the H chain V region of mouse ONS-M21 antibody contained in plasmid pUC-M21-V_H is shown in SEQ ID NO: 28 ~~27~~.

Example 3. Determination of CDR

The overall structures of the V regions of the L and H chains mutually resemble each other, and 4 framework regions are linked by 3 hypervariable regions, namely complementarity determining regions (CDRs). Although the amino acid sequence of the framework is relatively well preserved, the variability of the amino acid sequence of the CDRs is extremely high (Kabat, E.A. et al., "Sequences of Proteins of Immunological Interest", US Dept. of Health and Human Services, 1983).

On the basis of these facts, the CDRs were determined as shown in Table 4 as a result of investigating homology by applying the amino acid sequence of the variable region of mouse monoclonal antibody to human medulloblastoma cells to the database of antibody amino acid sequences prepared by Kabat, et al.

Table 4

Plasmid	SEQ ID NO	CDR(1)	CDR(2)	CDR(3)
pUC-M21-V_L	26	24-34	50-56	89-97
pUC-M21-V_H	29	31-35	50-66	99-106

Example 4. Confirmation of Expression of Cloned cDNA (Preparation of Chimeric ONS-M21 Antibody)

Preparation of Expression Vector

In order to make vectors that express chimeric ONS-M21 antibody, cDNA clone pUC-M21-V_L and pUC-M21-V_H that respectively code for the V regions of mouse ONS-M21κ L chain and H chain were modified by PCR. They were then introduced into HEF expression vectors (see, the previously mentioned WO92-19759) (see, Fig. 1).

Backward primer ONS-L722S (SEQ ID NO: 30) for the L chain V region and backward primer ONS-H3.2S (SEQ ID NO: 31) for the H chain V region were designed to hybridize with DNA coding for the start portion of the leader sequences of each V region and to have the Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and an HindIII restriction site. Forward primer ONS-L722A (SEQ ID NO: 32) for the L chain V region and forward primer ONS-H3.2A (SEQ ID NO: 33) for the H chain V region were designed to hybridize with DNA coding for the end portion of the J region and to have a splice donor sequence and BamHI restriction site.

100 µl of PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 µM dNTPs, 1.5 mM $MgCl_2$, 100 pmoles of each primer, 100 ng of template DNA (pUC-M21-V_L or pUC-M21-V_H) and 5 units of Ampli Taq were covered with 50 µl of mineral oil. Following initial denaturation at 94°C, an incubation cycle consisting of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C was repeated 30 times followed by final incubation for 10 minutes at 72°C.

The PCR products were purified using 5% low melting temperature agarose gel, digested with HindIII and BamHI, and the L chain V region was cloned into HEF expression

vector HEF-V_L-gκ, whil the H chain V r gion wa cloned into HEF expression vector HEF-V_H-gγ. After determining the DNA sequenc s, plasmids containing DNA fragments having the correct DNA sequenc w re resp ctively named HEF-M21L-gκ and HEF-M21H-gγ1.

Transfection into COS Cells

In order to observe the transient expression of chimeric ONS-M21 antibody, the above-mentioned expression vectors were tested in COS cells. HEF-M21L-gκ and HEF-M21H-gγ1 were co-transfected into COS cells by electroporation using a Gene Pulser apparatus (BioRad). Each DNA (10 μg) was added to an 0.8 ml aliquot of 1 x 10^7 cells/ml in PBS followed by the application of pulses at 1,900 V and capacitance of 25 μF.

After allowing a recovery period of 10 minutes at room temperature, the electroporated cells were added to DMEM culture medium containing 10% γ-globulin-free fetal calf serum (Gibco). After incubating for 72 hours, the culture supernatant was collected, cell debris was removed by centrifugation, and the supernatant was applied to a Protein A agarose column equilibrated with 5 volumes of binding buffer (Affi-Gel Protein A MAPSII Kit, BioRad). After washing the column with 15 volumes of binding buffer, it was eluted with 5 volumes of elution buffer. The eluate was concentrated and the buffer was changed to PBS using a microconcentrator (Centricon 100, Amicon).

Cell-ELISA

A Cell-ELISA plate for measuring antigen binding was prepared in the following manner. Human medulloblastoma cell line ONS-76 (Tamura, et al., Cancer Res., 49, 5380-5384 (1989)) prepared to 1 x 10^6 cells/ml with RPMI buffer containing 10% fetal calf serum was added to a 96-well plate. After culturing overnight, the cells were fixed with 0.1% glutaraldehyde (Nagai Chemical and Pharmaceuticals). After blocking, chimeric ONS-M21 antibody was serially diluted and added to each well. After incubating at room temperature and washing, alkaline

phosphatase-bound goat anti-human IgG antibody (Sigma) was added. After additional incubation and washing, substrate solution was added follow d by measurement of absorbance at 405 nm.

As a result, it was suggested that chimeric antibody ONS-M21 has the correct structure of the V region of mouse monoclonal antibody ONS-M21 since it specifically bound with medulloblastoma cell line ONS-76 (see, Fig. 2).

Example 5. Preparation of Reshaped Human ONS-M21 Antibody

Preparation of Reshaped Human ONS-M21 Antibody L Chain V Region

An L chain of a reshaped human ONS-M21 antibody was prepared by CDR-grafting using PCR. This technique is schematically illustrated in Fig. 3. In order to prepare a reshaped human antibody ONS-M21 having FRs derived from human antibody REI (version "a"), 8 PCR primers were used. External primers A (SEQ ID NO: 34 ~~32~~) and H (SEQ ID NO: 32 ~~33~~) were designed so as to hybridize with the DNA sequence of HEF expression vector HEF-V_L-gk.

CDR-grafting primers B (SEQ ID NO: 36 ~ ~~34~~), C (SEQ ID NO: 37 ~~35~~) and D (SEQ ID NO: 38 ~~36~~) have sense DNA sequences, while CDR-grafting primers E (SEQ ID NO: 39 ~ ~~37~~), F (SEQ ID NO: 40 ~~38~~) and G (SEQ ID NO: 41 ~ ~~39~~) have anti-sense DNA sequences, and have complementary DNA sequences (23-35 bp) to the DNA sequences of the 5'-terminals of primers B, C and D, respectively.

In the first stage of PCR, the four reactions between A-E, B-F, C-G and D-H were performed, and each PCR product was purified. The four PCR products from the first stage of PCR were assembled according to their own complementarity (see, WO92-19759). Next, external primers A and H were added to amplify the entire DNA coding for an L chain V region of a reshaped human ONS-M21 antibody (second stage of PCR). In the above-mentioned PCR, plasmid HEF-RV_L-SK2a coding for version "a" of the L chain V region of reshaped human SK2 antibody based on the FRs from human

antibody REI (se , Japanese Unexamined Patent Publication No. 5-129787) was used as a template.

In the first stage of PCR, 100 µl of PCR mixture containing 50 mM KCl, 100 µM dNTPs, 1.5 mM $MgCl_2$, 100 ng of template DNA, 100 pmoles of each prim r and 5 units of Ampli Taq were used. Each PCR tube was covered with 50 µl of mineral oil. After initially denaturing at 94°C, a reaction cycle was performed consisting of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C followed finally by incubation for 10 minutes at 72°C.

PCR products A-E (218 bp), B-F (101 bp), C-G (131 bp) and D-H (147 bp) were purified using 1.5% low melting temperature agarose gel, and assembled in the second stage of PCR. In the second stage of PCR, 98 µl of PCR mixture containing 1 µg of each first stage PCR product and 5 units of Ampli Taq were incubated in a cycle consisting of 2 minutes at 94°C, 2 minutes at 55°C and 2 minutes at 72°C after which 100 pmoles of each external primers (A and H) were added. The PCR tube was covered with 50 µl of mineral oil and 30 cycles of PCR were performed under the same conditions as described above.

The 516 bp DNA fragment produced from the second stage of PCR was purified with 1.5% low melting temperature agarose gel, digested with BamHI and HindIII, and the resulting DNA fragment was cloned into HEF expression vector HEF-V_L-gk. After determining the DNA sequence, the plasmid containing the DNA fragment coding for the correct amino acid sequence of the L chain V region of a reshaped human ONS-M21 antibody was named HEF-RVL-M21a-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21a-gK are shown in SEQ ID NO: 39 ~~40~~.

Version "b" of the L chain V region of reshaped human ONS-M21 antibody was prepared by PCR mutagenesis. Mutagenic primers FTY-1 (SEQ ID NO: 44 ~~41~~) and FTY-2 (SEQ ID NO: 45 ~~42~~) were designed so that the phenylalanine at position 71 is substituted to tyrosine.

After amplification using the above-mentioned primers and plasmid HEF-RVL-M21a-gκ as a t mplate, the final product was purified, digested with BamHI and HindIII, and the resulting DNA fragment was cloned into HEF expression vector HEF-VL-gκ to obtain plasmid HEF-RVL-M21b-gκ. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21b-gκ are shown in SEQ ID NO: 43.

Each of versions "c", "d", "e", "f", "g", "h", "i", "j", "k", "l", "m", "n", "o" and "p" of the L chain V region of reshaped human ONS-M21 antibody was produced in the manner described below.

For version "c", amplification was performed by PCR using primers M21M2S (SEQ ID NO: 48) and M21M2A (SEQ ID NO: 49), which were designed so that tyrosine at position 87 is substituted to phenylalanine, as mutagenic primers, and using plasmid HEF-RVL-M21a-gκ as a template DNA to obtain plasmid HEF-RVL-M21c-gκ. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21c-gκ are shown in SEQ ID NO: 50 and 51.

For version "d", amplification was performed using primers FTY-1 and FTY-2 as well as M21M2S and M21M2A as mutagenic primers, and using plasmid HEF-RVL-M21a-gκ as a template DNA to obtain plasmid HEF-RVL-M21d-gκ. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21d-gκ are shown in SEQ ID NO: 52 and 53.

For version "e", amplification was performed using primers M21M3S (SEQ ID NO: 54) and M21M3A (SEQ ID NO: 55), which were designed so that threonine at position 20 is substituted to serine and isoleucine at position 21 is substituted to valine, as mutagenic primers, and using plasmid HEF-RVL-M21a-gκ as a template DNA to obtain plasmid HEF-RVL-M21e-gκ. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21e-gκ are shown in SEQ ID NO: 56 and 57.

For version "f", plasmid HEF-RVL-M21c-gK was digested with BamHI and HinfI, and plasmid HEF-RVL-M21e-gK was digested with HindIII and HinfI to obtain 152 bp and 250 bp DNA fragments respectively. After isolating and purifying these DNA fragments using 15% low melting temperature agarose gel, the fragments were linked and inserted into HEF expression vector HEF-RVL-gK to obtain plasmid HEF-RVL-M21f-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21f-gK are shown in SEQ ID NO: 58 and 59 ~~51~~.

For version "g", amplification was performed using primers M21M4S (SEQ ID NO: 60 ~~52~~) and M21M4A (SEQ ID NO: 61 ~~53~~), which were designed so that phenylalanine at position 73 is substituted to leucine, as mutagenic primers, and using plasmid HEF-RVL-M21f-gK as a template DNA to obtain plasmid HEF-RVL-M21g-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21g-gK are shown in SEQ ID NO: 62 and 63 ~~54~~.

For version "h", amplification was performed using primers M21M4S and M21M4A as mutagenic primers, and using plasmid HEF-RVL-M21a-gK as a template DNA to obtain plasmid HEF-RVL-M21h-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21h-gK are shown in SEQ ID NO: 64 and 65 ~~55~~.

For version "i", amplification was performed using primers M21M5S (SEQ ID NO: 66 ~~56~~) and M21M5A (SEQ ID NO: 67 ~~57~~), which were designed so that lysine at position 42 is substituted to glutamine, analine at position 43 is substituted to serine and leucine at position 46 is substituted to proline, as mutagenic primers, and using plasmid HEF-RVL-M21g-gK as a template DNA to obtain plasmid HEF-RVL-M21i-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21i-gK are shown in SEQ ID NO: 68 and 69 ~~58~~.

For version "j", amplification was performed using primers M21M5S, M21M5A as well as M21M6S (SEQ ID NO: 70 ~~59~~) and M21M6A (SEQ ID NO: 71 ~~60~~), which were designed so that

thr onine at position 85 is substituted to aspartate, as mutagenic primers, and using plasmid HEF-RVL-M21i-gK as a templat DNA to obtain plasmid HEF-RVL-M21j-gK. The amino acid sequ nc and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21j-gK are shown in SEQ ID NO: 72 and 73.

For version "k", amplification was performed using primers M21M6S and M21M6A as mutagenic primers, and using plasmid HEF-RVL-M21g-gK as a template DNA to obtain plasmid HEF-RVL-M21k-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21k-gK are shown in SEQ ID NO: 74 and 75.

For version "l", plasmid HEF-RVL-M21a-gK was digested with BamHI and SfaNI, and plasmid HEF-RVL-M21i-gK was digested with HindIII and SfaNI to obtain 227 bp and 169 bp DNA fragments respectively. After isolating and purifying these DNA fragments, the resulting DNA fragments were linked and inserted into HEF expression vector HEF-RVL-gK to obtain plasmid HEF-RVL-M21l-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21l-gK are shown in SEQ ID NO: 76 and 77.

For version "m", amplification was performed using primers M21M5S, M21M5A as well as M21M7S (SEQ ID NO: 78) and M21M7A (SEQ ID NO: 79), which were designed so that proline at position 8 is substituted to glutamate, serine at position 9 is substituted to lysine and serine at position 10 is substituted to phenylalanine, as mutagenic primers, and using plasmid HEF-RVL-M21a-gK as a template DNA to obtain plasmid HEF-RVL-M21m-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21m-gK are shown in SEQ ID NO: 80 and 81.

For version "n", amplification was performed using primers M21M8S (SEQ ID NO: 82) and M21M8A (SEQ ID NO: 83), which were designed so that lysine at position 42 is substituted to glutamate and alanine at position 43 is

substituted to serine, as mutagenic primers, and using plasmid HEF-RVL-M21a-gK as a t mplate DNA to obtain plasmid HEF-RVL-M21n-gK. The amino acid sequence and nucleotid sequence of the L chain V region contain d in this plasmid HEF-RVL-M21n-gK are shown in SEQ ID NO: 84 and 85.

For version "o", plasmid HEF-RVL-M21b-gK was digested with BamHI and BsrI, and plasmid HEF-RVL-M21a-gK was digested with HindIII and BsrI to obtain 251 bp and 142 bp DNA fragments respectively. After isolating and purifying these DNA fragments, the resulting DNA fragments were linked and inserted into HEF expression vector HEF-RVL-gK to obtain plasmid HEF-RVL-M21o-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21o-gK are shown in SEQ ID NO: 86 and 87.

For version "p", amplification was performed using primers M21M9S (SEQ ID NO: 88) and M21M9A (SEQ ID NO: 89), which were designed so that leucine at position 46 is substituted to proline, as mutagenic primers, and using plasmid HEF-RVL-M21a-gK as a template DNA to obtain plasmid HEF-RVL-M21p-gK. The amino acid sequence and nucleotide sequence of the L chain V region contained in this plasmid HEF-RVL-M21p-gK are shown in SEQ ID NO: 90 to 93.

Preparation of Reshaped Human ONS-M21 Antibody H Chain V Region

DNA coding for the H chain V region of reshaped human ONS-M21 antibody was designed in the following manner. DNA sequences coding for FR1-3 of human antibody Eu and FR4 of human antibody ND were designed based on "codon usage" of the V region (Kabat, E.A. et al., US Dept. of Health and Human Services, US Government Printing Offices, 1991). By connecting with a DNA sequence coding for the CDRs of the H chain V region of mouse ONS-M21 antibody, an entire length of DNA was designed coding for the H chain V region of reshaped human ONS-M21 antibody.

Next, a HindIII recognition site/KOZAK consensus sequence and BamHI recognition site/splice donor sequence

were added to this 5'-terminal and 3'-terminal, respectively, of this DNA sequence to enable inserted into an HEF expression vector.

The DNA sequence designed in this manner was then divided into four oligonucleotides, and the secondary structures of oligonucleotides having the potential to inhibit assembly of these oligonucleotides were analyzed by computer.

The four oligonucleotide sequences are shown in SEQ ID NO: 93 to 97. These oligonucleotides have lengths of 111 to 137 bases, and possess overlapping regions of 23 to 26 bp. Among the oligonucleotides, the RVH2 (SEQ ID NO: 94) and RVH4 (SEQ ID NO: 95) have sense DNA sequences, while the other RVHI (SEQ ID NO: 96) and RVH3 (SEQ ID NO: 97) have anti-sense DNA sequences. The assembly method of these four oligonucleotides by PCR is shown in the illustration (see, Fig. 4).

After initially denaturing 98 µl of PCR mixture containing 100 ng of each of the four types of oligonucleotides and 5 units of Ampli Taq for 2 minutes at 94°C, the mixture was incubated for 2 cycles consisting of 2 minutes at 94°C, 2 minutes at 55°C and 2 minutes at 72°C. After adding 100 pmoles each of RHP1 (SEQ ID NO: 98) and RHP2 (SEQ ID NO: 99) as external primers, the PCR tube was covered with 50 µl of mineral oil, and after initially denaturing for 1 minute at 94°C, 38 cycles of incubation consisting of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C were performed followed by final incubation for 10 minutes at 72°C.

The 438 bp DNA fragment was purified using 1.5% low melting temperature agarose gel, digested with HindIII and BamHI, and cloned in HEF expression vector HEF-V_H-gγ1. After determining the DNA sequence, the plasmid containing the DNA fragment coding for the correct amino acid sequence of the H chain V region was named HEF-RVH-M21-gγ1. The amino acid sequence and nucleotide sequence of the H chain

V region contained in this plasmid HEF-RVH-M21-gγ1 are shown in SEQ ID NO: 98 and 99.

In order to evaluate each chain of reshaped human ONS-M21 antibody, COS cells were co-transfected as previously described with expression vector HEF-RVH-M21-gγ1 for the H chain of reshaped human ONS-M21 antibody, and expression vector HEF-M21L-gγ1 for the L chain of chimeric ONS-M21 antibody. After collecting the antibody products as previously described, antigen binding was measured as previously described.

Those results are shown in Fig. 5. As shown in Fig. 5, there was confirmed to be no difference in antigen binding between chimeric antibody (ChM21) used as positive control and antibody comprising reshaped H chain and chimeric L chain (ChL/RVH).

Next, in order to evaluate combinations of each of versions "a" through "p" of reshaped humanized ONS-M21 antibody L chain and reshaped humanized ONS-M21 antibody H chain, one of each of the expression plasmids from HEF-RVL-M21a-gK to HEF-RVH-M21p-gK of each version of the L chain and H chain expression plasmid HEF-RVH were co-transfected into COS cells and antigen binding was measured for the resulting antibodies using the method as described in Cell ELISA of the above-mentioned Embodiment 4. As a result, antigen binding activity was not observed in antibodies having L chain versions "a" through "h" (see, Fig. 6).

On the other hand, antibodies having each of the L chain versions "i", "j", "l", "m", "o" and "p" demonstrated good antigen binding comparable to that of the positive control chimeric ONS-M21 antibody (ChM21), and this combination was suggested to create a functional antigen binding site in human antibody. Furthermore, antigen binding activity was not observed in antibodies having L chain versions "k" and "n" (refer to Fig. 7).

On the basis of these findings, antibody having proline at position 46 of the L chain FR2 was suggested to

recreate a functional antigen binding site that exhibits good antigen binding.

Example 6. Preparation of Reshaped Human ONS-M21 Antibody Single-Chain Fv (scFV)

Construction of Linker Region

DNA coding for a linker region consisting of Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO: 114) was designed in the following manner. A 15 bp DNA sequence coding for 5 amino acid residues of the C-terminal of FR4 of the H chain V region was added to the 5'-terminal of a DNA sequence coding for a linker region (Huston, J.S. et al., Proc. Natl. Acad. Sci. USA, 85, 5879-5883 (1988)), while a 15 bp DNA sequence coding for 5 amino acid residues of the N-terminal of the FR1 region of the L chain V region was added to the 3'-terminal. Moreover, HindIII and EcoRI recognition sites were added to the 5'-terminal and 3'-terminal, respectively, to enable insertion into pUC19 vector.

Two oligonucleotides scFv-S and scFv-A in the sense and anti-sense directions were synthesized based on the DNA sequences designed in this manner.

The two oligonucleotide sequences are shown in SEQ ID NOs: 81 and 82, respectively. These oligonucleotides have a length of 84 bases, and have an overlapping region of 81 bp.

100 pmoles each of the two oligonucleotides were placed in 20 µl of a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 50 mg/ml polyethylene glycol (8000). Annealing was performed by incubating for 5 minutes at 96°C, lowering the temperature to 65°C over the course of 20 minutes, incubating for 10 minutes at 65°C, lowering the temperature to 37°C over the course of 20 minutes, incubating for 10 minutes at 37°C, lowering the temperature to 7°C over the course of 20 minutes and finally incubating at 7°C overnight.

The DNA annealed in the above-mentioned mann r w s inserted into pUC19 vector cleaved with HindIII and EcoRI. After determining the DNA sequence, the plasmid containing the DNA fragm nt that has the correct DNA sequence was nam d pUC-scFv-5.

Preparation of Reshaped Human ONS-M21 Antibody Single-Chain Fv

The single-chain Fv of reshaped human ONS-M21 antibody was produced in the following manner. Reshaped human ONS-M21 antibody H chain V region, linker region and reshaped human ONS-M21 antibody L chain V region were respectively amplified and assembled using PCR to construct reshaped human ONS-M21 antibody single-chain Fv. This procedure is schematically illustrated in Fig. 8. Six PCR primers (A to F) were used to construct the single-chain Fv of reshaped human ONS-M21 antibody. Primers A, C and E have sense sequences, while primers B, D and F have anti-sense sequences.

Backward primer SCP1 (primer A, SEQ ID NO: 83) for the H chain V region was designed to hybridize with DNA coding for the N-terminal of the H chain V region and to have an NcoI recognition site. Forward primer SCP2 (primer B, SEQ ID NO: 84) for the H chain V region was designed to hybridize with DNA coding for the C-terminal of the H chain V region and to overlap with the linker. Backward primer SCP3 (primer C, SEQ ID NO: 85) for the linker region was designed to hybridize with DNA coding for the N-terminal of the linker and to overlap with DNA coding for the C-terminal of the H chain V region.

Forward primer SCP4 (primer D, SEQ ID NO: 86) for the linker region was designed to hybridize with DNA coding for the C-terminal of the linker and to overlap with DNA coding for the N-terminal of the L chain V region. Backward primer SCP4 (primer E, SEQ ID NO: 87) for the L chain V region was designed to hybridize with DNA coding for the C-terminal of the linker and to overlap with DNA coding for the N-terminal of the L chain V region. Forward primer

WS4-2 (primer F, SEQ ID NO: ~~88~~ 107) for th L chain V region was d signed to hybridiz with DNA coding for the C-terminal of th L chain V region and to have a sequence coding for the FLAG peptide (Hopp, T.P. et al., Bio/Technology, 6, 1204-1210, 1988), two transcription termination codons and an EcoRI recognition site.

In the first step of PCR, three reactions were conducted between A-B, C-D and E-F and each PCR product was purified. The three PCR products from the first step of PCR were assembled according to their own complementarity. Next, primers A and F were added to amplify the entire length of DNA coding for the single-chain Fv of reshaped human ONS-M21 antibody (second step of PCR). Furthermore, in the first step of PCR, plasmid HEF-RVH-M21-gγ1 coding for the H chain V region of reshaped human ONS-M21 antibody (refer to Embodiment 5), plasmid pUC-scFv-5 coding for the linker region, and plasmid HEF-RVL-M21p-gκ (refer to Embodiment 5) coding for version "p" of the L chain V region of reshaped human ONS-M21 antibody were respectively used as templates.

In the first step of PCR, a PCR mixture was used containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 μM dNTPs, 1.5 mM $MgCl_2$, 100 ng of each template DNA, 100 pmoles of each primer and 5 units of DNA polymerase Ampli Taq (Perkin Elmer Cetus). After covering each PCR tube with 50 μl of mineral oil, the tube was heated in the order of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C. After repeating this temperature cycle 30 times, the reaction mixture was additionally incubated for 10 minutes at 72°C.

PCR products A-B (382 bp), C-D (92 bp) and E-F (363 bp) were purified using 1.5% low melting temperature agarose gel and assembled in the second step of PCR. In the second step of PCR, 98 μl of PCR reaction mixture, containing 1 μg of each of the first stage PCR products as templates and 5 units of Ampli Taq, were incubated for 2 cycles consisting of 2 minutes at 94°C, 2 minutes at 55°C

and 2 minutes at 72°C followed by addition of 100 pmoles each of primers A and F. After covering th PCR tub with 50 µl of mineral oil, 30 cycles of PCR were performed consisting of 1 minute at 94°C, 1 minute at 55°C and 2 minutes at 72°C.

The 767 bp DNA fragment produced in the second step of PCR was purified with 1.5% low melting temperature agarose gel and digested with NcoI and EcoRI, after which the resulting DNA fragments were cloned in expression vector pSCFVT7. Furthermore, this expression vector pSCFVT7 contains a pelB signal sequence suited to E. coli periplasm secretion expression systems (Lei, S.P. et al., J. Bacteriology, 169, 4379-4383, 1987). After determining the DNA sequence, the plasmid containing the DNA fragment coding for the correct amino acid sequence of the single-chain Fv of reshaped human ONS-M21 antibody was named pSCFVT7-hM21 (see Fig. 9). The amino acid sequence and nucleotide sequence of the single-chain Fv of reshaped human ONS-M21 antibody contained in this plasmid SCFVT7-hM21 are shown in SEQ ID NO: 108 and 109 89.

Transformation of E. coli strain BL21(DE3)

10 ng of the above-mentioned plasmid pSCFVT7-hM21 was added to 50 µl of E. coli BL21(DE3) competent cells, after which the cells were allowed to stand for 30 minutes on ice, for 90 seconds at 42°C and again for 1 minute on ice. Next, 400 µl of 2 x YT medium was added and after incubating for 1 hour at 37°C, the E. coli was plated onto 2 x YT agar medium and incubated overnight at 37°C to obtain E. coli transformants.

Induction of Expression of Reshaped Human ONS-M21 Antibody Single-Chain Fv Region

The transformed E. coli was cultured overnight at 37°C in 30 ml of LB medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) containing 1% glucose and 50 µg/ml ampicillin. Next, the culture was innoculated into 100 times volume of LB medium containing 50 µg/ml ampicillin and cultured at

37°C. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM when absorbance at 650 nm reached about 0.3 to induce expression from T7 promoter.

After additionally culturing overnight at 37°C, the medium was collected, cell debris was removed by centrifugation followed by the addition of an equal volume of PBS. After equilibrating with 15 ml of 0.1 M glycine-HCl (pH 3.0), the medium was applied to an anti-FLAG affinity column neutralized with an equal volume of PBS (Anti-FLAGM2 Affinity Gel, IBI). After washing the column with 3 volumes of PBS, the column was eluted with 6 ml of 0.1 M glycine-HCl (pH 3.0). The eluate was concentrated and the buffer was changed to PBS using a microconcentrator (Centricon10, Amicon).

Cell-ELISA

The antigen binding activity of the single-chain Fv of reshaped human ONS-M21 antibody was measured using for an indicator an inhibitory activity of mouse monoclonal ONS-M21 antibody on antigen binding. After blocking the Cell-ELISA plate prepared as described above, serially diluted samples of the reshaped human ONS-M21 antibody single-chain Fv, reshaped human ONS-M21 antibody or of Fab fragment prepared from the reshaped human ONS-M21 antibody were added together with mouse monoclonal ONS-M21 antibody at a concentration of 500 ng/ml to each well. After incubating at room temperature and washing, alkaline-phosphatase-bound goat anti-mouse IgG antibody (Zymed) was added. After incubating and washing, substrate solution was added followed by measurement of absorbance at 405 nm.

As a result, although inhibitory activity of the single-chain Fv of reshaped human ONS-M21 antibody (scFv-hM21) decreased to about 1/10 in comparison with reshaped human ONS-M21 antibody (hM21), it showed almost same inhibitory activity as the Fab fragment prepared from reshaped human ONS-M21 antibody (see, Fig. 10).

On the basis of these findings, single-chain Fv of a reshaped human ONS-M21 antibody was suggested to exhibit

roughly th same degree of affinity as the original reshaped human ONS-M21 antibody.

Furthermore, E. coli having the above-mentioned plasmid HEF-RVL-M21p-gκ and E. coli containing plasmid HEF-RVH-M21-gγ1 are respectively deposit d as Escherichia coli DH5α (HEF-RVL-M21p-gκ) and Escherichia coli DH5α (HEF-RVH-M21-gγ1) at the National Institute of Bioscience and Human Technology Agency of Industrial Science and Technology (1-3 Higashi, 1-chome Tsukuba-shi, Ibaraki), and were submitted for international deposit under the provisions of the Budapest Accord as FERM BP-4472 and FERM BP-4471 on November 18, 1993.

Reference Example 1. Preparation of Hybridoma ONS-M21

Hybridoma that produces an anti-human medulloblastoma cell monoclonal antibody was prepared by fusing spleen cells of a BALB/c mouse immunized with human medulloblastoma cells ONS-76 and mouse myeloma cells P3U1 in accordance with routine methods using polyethylene glycol. Screening was performed using as an indicator a binding activity with medulloblastoma cells ONS-76 so as to establish hybridoma ONS-M21 (Moriuchi, S. et al., Br. J. Cancer, 68, 831-837 (1993)).

Reference Example 2. Typing of Mouse Monoclonal Antibody ONS-M21

Hybridoma ONS-M21 was transplanted into mouse abdominal cavity and the resulting ascites was applied to a Protein A agarose column to obtain purified mouse monoclonal antibody. In order to investigate the types of the L chain and H chain of the resulting mouse monoclonal antibody ONS-M21, typing was performed using a mouse monoclonal antibody isotyping kit (Amersham International Plc.). As a result, ONS-M21 antibody was clearly shown to have a κ type L chain and γ1 type H chain.

Reference to Microorganisms Deposited Based on Provision 13, Part 2 of the Budapest Treaty:

Deposition Institution: National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology

Address: 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki

Deposition Numbers and Dates:

1. Escherichia coli DH5α (HEF-RVL-M21p-gκ)
   Deposition no.: FERM BP-4472
   Deposition date: November 18, 1993
2. Escherichia coli DH5α (HEF-RVH-M21-gγ1)
   Deposition no.: FERM BP-4471
   Deposition date: November 18, 1993